DESCRIPTION

Rotary Motor Molecule V_1-ATPase

Technical Field

The present invention relates to a novel rotary motor molecule V_1-ATPase useful for a nanoactuator of a micromachine or a nanomachine, or the like.

Background Art

Attention is being given to the development of a micromachine or a nanomachine that mechanically moves according to the size of a molecule. This is because such micromachine or nanomachine is considered useful for, e.g., a molecule robot that lays out the wiring of a molecule computer or a medical robot that works a cure in the body.

For the fabrication of a micromachine and a nanomachine, development of a variety of technologies is required, including individual element devices (a sensor, an actuator and a miniature machine) to processes of assembling them (micromachining and nanomaching). In particular, the development of microactuators and nanoactuators (rotary motors), i.e., micromachine drive devices, is essential for self-regulating movement of machines, and the development of motor devices utilizing diverse precise handling technologies is being pursued. However, even microactuators made by processes to which precise handling technologies

are applied are no smaller than about 100 μm. Further miniaturization of motor apparatuses is being required to install them in micromachines and nanomachines.

Thus, aside from construction of a motor by precise handling technology, utilization of a single molecule having rotary movement capability as a motor is proposed.

In general, a molecule capable of being a motor needs to satisfy two factors: having a power mechanism that converts outer energy into rotary movement, and achieving rotation in one direction. Low molecular organic compounds satisfying such conditions that are known include, for example, (3R,3'R)-(P,P)-trans-1,1',2,2',3,3',4,4'-octahydro-3,3'-dimethyl-4,4'-bipheant hrydiene (Nature 401: 152-155, 1999) and Triptycyl(4)helicene (Nature 401: 150-152, 1999). The former has symmetry to the right and to the left of the carbon-carbon double bond, but has a twisted structure due to steric interlocking. Addition of suitable heat or light thereto makes it possible to rotate the molecule in one direction through four process steps. Also, one cycle is completed through two light reactions and a heat isomerization, with the movement proceeding in one direction only. In other words, this organic compound conducts rotary motion via heat isomerization and light reaction. Rotation via light reaction is very rapid (a level of picoseconds), but rotation via heat isomerization needs a few minutes, and so is unsuitable for actual use. Furthermore, the compound poses the problem that the driving force of rotation is extremely weak. On the other hand, the heat isomerization causes one-direction rotation of the molecule utilizing the chemical reactions of phosgene addition and the formation and cleavage of urethane bonding. However, this molecule is incapable of repeating rotation, a fatal defect for an actuator.

On the other hand, as a single molecule motor capable of being utilized in a micromachine, a nanomachine or the like, biomolecules are known that include a flagellum motor (Microbiol. 6: 1-18, 1967, Nature 245: 380-382, 1973), an ATP synthase (Nature 386: 299-302, 1997), a myosin motor (Biochem. Biophys. Res. Comm. 199: 1057-1063, 1994, Curr. Opin. Cell Biol. 7: 89-93, 1995), a microtubule-based motor (Cell 42: 39-50, 1985), a motor protein of nucleic acid synthase (Nature 409: 113-119, 2001), and the like.

Of these, an ATP synthase is a membrane protein present ubiquitous, at such locations as the inner membranes of mitochondria in eukaryotes, thylakoid membranes of chloroplasts, a prokaryote cell membrane, and the like, and synthesizes most ATP consumed in cells. An ATP synthase (F_0F_1-ATP synthase) is a huge membrane protein complex with molecular weight up to about 500 thousand, and consists of an F_0 portion present inside a membrane and an F_1 portion present outside the membrane. The F_0 portion is a passage for a proton (H^+) to pass through the membrane, and the F_1 portion is a catalyst portion that synthesizes and hydrolyzes ATP. The molecular weight of the F_1 portion is about 380 thousand, for example, the subunit composition of the F_1 portion in an ATP synthase derived from bacteria is $\alpha_3\beta_3\delta\gamma_1\varepsilon_1$. α and β subunits both have a similar ATP binding portion, but catalyst activity is present in the β subunit. Both alternately align to form a ring and in the center of this $\alpha_3\beta_3$ ring, a γ subunit is present. A δ subunit binds to the top of the $\alpha_3\beta_3$ ring; an ε subunit that controls ATP hydrolysis activity binds to the γ subunit. On the other hand, the F_0 portion has a molecular weight of about 100 thousands, and the amino acid composition contains in quantity glutamic acid and asparaginic acid, necessary for proton movement. The subunit composition is $a_1b_2c_{9\text{-}12}$, "c" subunits are arranged like a ring (the "c" ring) in the membrane, and to the "c" ring are bound subunit "a" and

two "b" subunits each having an arm protruding far outside the membrane. Hence, an F_0F_1-ATP synthase has an F_1 portion and an F_0 portion which are bound to each other at two sites: γ ε -"c" ring and δ b_2. A further characteristic is the fact that this F_0F_1-ATP synthase molecule has two kinds of torque generating devices. One is an ATP driving type device present in the F_1 portion and the other is a proton driving type device present in the F_0 portion. That is, when the F_0 portion takes a proton in the cell membrane, the "c" ring rotates clockwise; when the F_0 portion discharges a proton to the outside of the cell membrane, the "c" ring rotates anticlockwise. On the other hand, during ATP synthesis, the F_1 portion rotates clockwise viewing the γ subunit from the F_0 side, and the F_1 portion rotates anticlockwise during ATP decomposition. By providing these two kinds of torque generating devices, the torque generated by ATP synthase is on the order of tens of piconewton·nm, and thus the synthase has a sufficient driving force for a molecule motor. Additionally, an ATP synthase acts in a water system and so it is most suitable as an actuator working in the body, and also can manipulate a protein, sugar, a lipid, or a nucleic acid in the body because it has sufficient power for moving actin.

The inventors of the present invention improved this F_0F_1-ATP synthase molecule, and have already invented and filed the invention of a modified F_0F_1-ATP synthase molecule capable of controlling over a wide rotation speed range and its utilization (Japanese Patent Application No. 2002-148232; filing date: May 22, 2002). In addition, recently, reported was a rotary motor molecule, which is made by incorporating a zinc binding site into an F_1-ATP synthase molecule and which is capable of controlling the initiation and stop of the rotation by means of the zinc (Nature Materials 1: 173-177, 2002).

As described above, various rotary motor molecules are proposed as

driving members of a micromachine, a nanomachine, and the like, and the molecules each have characteristics regarding type of rotation, the revolution number, torque, the method of controlling rotation, etc. Accordingly, for actual fabrication of a micromachine or a nanomachine, an appropriate molecule needs to be selected from a variety of candidate molecules depending on its application and machine construction. However, it cannot be said that the rotary motor molecules reported thus far can each be suitable for all the different applications and constructions of a micromachine and a nanomachine. For this reason, upon the development of a micromachine or a nanomachine or the like, each addition of one more to the lineup of rotary motor molecules is greatly desired.

Thus, this application is intended to provide a novel rotary motor molecule that is different in properties from the conventional rotary motor molecules.

In addition, the application also has another subject of providing an improved, novel rotary motor molecule which further smoothens the rotary motion and also adds a means for the molecule to transfer the rotary motion.

Disclosure of Invention

This application, as the invention for solving the above-described problem, provides a rotary motor molecule V_1-ATPase rotating in the presence of ATP, which is a complex molecule having three A subunits, three B subunits and one D subunit constituting the V_1 portion of a V_0V_1-ATPase.

The V_1-ATPase of this invention includes the A subunit as a catalyst portion, A and B subunits are arranged alternately, and from a hexamer cylinder like the $\alpha_3\beta_3$ of a V_0V_1-ATPase. The D subunit is embedded in the central cavity of this A_3B_3 cylinder, an F subunit is bound to the D subunit, and the D and F subunits act as a rotor (rotary shaft, rotary axis).

In one mode of this invention, the rotary motor molecule V_1-ATPase is heat resistant, and in this case it is preferred that the V_1-ATPase is derived from the thermophile bacteria, *Thremus thermophilus*.

Further, the rotary motor molecule V_1-ATPase derived from the thermophile, *Thremus thermophilus* is a complex having three peptides of SEQ ID NO:3 corresponding to the A subunit, three peptides of SEQ ID NO:4 corresponding to the B subunit, and one peptide of SEQ ID NO:5 corresponding to the D subunit is one preferred mode.

Furthermore, in another mode of the invention, the rotary motor molecule V_1-ATPase of the present invention is an improved type molecule which has at least one substitution of Ala residue for the 232nd Ser residue and Ser residue for the 235th Thr residue in SEQ ID NO:3.

In this improved V_1-ATPase, the improvement of the A subunit, the catalyst portion, allows dissolution of MgADP inhibition and acceleration of ATP hydrolysis activity. Rotation of a wild type of V_1-ATPase tends to be suppressed due to MgADP inhibition, and the modified V_1-ATPase that prevents MgADP inhibition exhibits efficient rotational motion.

Furthermore, a further mode of the present invention is that the

rotary motor molecule V_1-ATPase is an improved molecule in which one or both of the A and B subunits is fixed on a substrate. In this case, a preferred mode is that the molecule is fixed on the substrate via an His tag bound to the N terminal of the A subunit.

In a still another mode of the invention, the rotary motor molecule V_1-ATPase has a joint bound to the D subunit. In this case, the joint material is bound to at least one of Cys residue substituted for the 48th Glu residue and Cys residue substituted for the 55th Gln residue in SEQ ID NO:5, and the case where all the Cys residues in the A and B subunits are replaced by non-Cys residues is another preferred mode.

That is, the rotary motor molecule V_1-ATPase of the present invention comprises the V_1 portion (a complex comprised of three A subunits, three B subunits, one D subunit) of V type (tonoplast type) ATPase (V_0V_1-ATPase) present in organelles (vacuole, lysosome, Golgi vesicle, cell membrane, coated vesicle, secretory granule, etc) of a bacterium or a eukaryote. Although a V_0V_1-ATPase synthase has already known to serve as a rotary motor molecule, the V_1 portion (V_1-ATPase) of this V_0V_1-ATPase was not known to have rotational motion at all. The V_1-ATPase of this invention has been completed by for the first time finding that the D subunit located inside a "cylindrical body" comprised of three A subunits and three B subunits a D subunit functions as a rotary shaft.

In addition, the V_1 portion of a V_0V_1-ATPase has one F subunit bound to the D subunit, but the V_1-ATPase of this invention also includes a molecule that binds this F subunit. Also, examples of V_1-ATPases of this invention include not only a wild type, but also a variety of variants as described above. Furthermore, the examples include a variant into which a zinc recognizing portion is incorporated, as disclosed in Nature Materials

1: 173-177, 2002 as noted above.

Hereinafter, each invention as described above will be set forth in detail with embodiments of the invention. For the embodiments, a variety of arts used in order to carry out this invention, exclusive of the arts particularly indicating the sources thereof, can be readily and surely carried out by a person skilled in the art in accordance with literatures or the like. For instance, descriptions of genetic engineering and molecular biological technology, such as Sambrook and Maniatis, in Molecular Cloning-A Laboratory Manual, Cold Spring Harbor Laboratory Press, New York, 1989; and Ausubel, F. M. et al., Current John Wiley & Sons, New York, N. Y. Protocols in Molecular Biology, 1995 can be used for reference.

Brief Description of Drawings

Figure 1 is a schematic diagram indicating the observed rotation of V_1-ATPase. The arrow indicates the direction of rotation.

Figure 2 is the result of western blotting analysis that has confirmed the biotinylation of a D or an F subunit. The left side (lanes 1 to 4) is obtained by CBB staining and the right side (lanes 5 to 8) is obtained by alkaline phosphatase-streptavidine conjugate staining. Lanes 1 and 5 indicate V_1-ATPase in which the D subunit was biotinylated, lanes 2 and 6 indicate V_1-ATPase with a biotinylated F subunit, lanes 3 and 7 indicate un-biotinylated V_1-ATPase, and lanes 4 and 8 indicate molecular-weight markers.

Figure 3 shows the measurements of rotation of the beads fixed on D subunits over time. "A" shows the rotation of bead in the presence of 4

mM ATP and 0.5 mM sodium azide. "B" to "D" show the results of rotations of beads in the absence of sodium azide, where "B" is 4 mM ATP solution, "C" is 0.5 mM ATP solution, and "D" is 0.2 mM ATP solution.

Figure 4 indicates the measurements of rotation of bead fixed on an F subunit in 4mM ATP solution.

Best Mode for Carrying Out the Invention

The rotary motor molecule V_1-ATPase of this invention is the V_1 (V_1-ATPase) portion of V_0V_1-ATPase produced from various bacteria or eukaryotes. The V_1-ATPase can be produced by genetic engineering using a polynucleotide (DNA fragment, RNA fragment, or preferably cDNA fragment. Hereinafter, it may be denoted as "V_1-ATPase polynucleotide") encoding the V_1−ATPase. Namely, sequences of the polynucleotide (cDNA fragment) encoding the V_0V_1-ATPase are disclosed in many data bases (e.g., GenBank data base: URL: http://www. ncbi. nlm. nih. gov), and using the sequence information in a probe hybridization process or a PCR process, the polynucleotide (cDNA fragment) encoding the V_0V_1-ATPase can be readily obtained from existent cDNA libraries, or the like.

Expression of this V_1-ATPase polynucleotide using a well-known genetic engineering process can provide a complex V_1-ATPase comprised of three A subunits, three B subunits and one D subunit. For instance, the recombination of a V_1-ATPase polynucleotide into an expression vector having an RNA polymerase promoter and then addition of this recombinant vector into an in vitro translation system including RNA polymerase corresponding to the promoter, such as rabbit reticlocyte lysate or a wheat embryo extract, can produce the V_1-ATPase having rotation capability *in*

vitro. Examples of the RNA polymerase promoter can include T7, T3, and SP6. Examples of vectors containing these RNA polymerase promoters include pKA1, pCDM8, pT3/T7 18, pT7/3 19, and pBluescript II. Also, the expression of a V_1-ATPase polynucleotide in a suitable host-vector system can produce the rotary motor molecule V_1-ATPase in a prokaryotic cell such as E. coli., or hay bacillus, a eukaryotic cell such as yeast, an insect cell, a mammal cell, or plant cell, or the like. For example, when the V_1-ATPase is expressed in a microorganism such as E. coli., the polynucleotide is recombined into an expression vector having an origin replicable in the microorganism, a promoter, a ribosome binding portion, a DNA cloning portion, a terminator and the like, to prepare an expression vector which transforms the host cell. Culturing this transformant can produce the target V_1-ATPase molecules from the culture in quantity. Examples of expression vector for E. coli include pUC system, pBluescript II, pET expression system, and pGEX expression system. Furthermore, when the polynucleotide is to be expressed in a eukaryotic cell, the polynucleotide is inserted into an expression vector for a eukaryotic cell, the vector having a promoter, a splicing region, poly(A) addition portion and the like, resulting in a recombinant vector. From eukaryotic cells transfected with this vector can be obtained the target V_1-ATPase molecules. Examples of the expression vector include pKA1, pCDM8, pSVK3, pMSG, pSVL, pBK-CMV, pBK-RSV, EBV vector, pRS, and pYES2. Eukaryotic cells that can be used include mammal culture cells such as human embryo renal cell line HEK293, monkey renal cell line COS7, Chinese hamster ovarian cell line CHO, or primary culture cells isolated from human organ, and the like. The eukaryotic cells that can be used also include budding yeast, fission yeast, silkworm cells, and Xenopus egg cells. For the expression vector to be transfected into eukaryotic cells, a known method such as electroporation, calcium phosphate method, ribosome method, DEAE dextran method, and the like may be used. For

isolation and purification of the V_1-ATPase expressed from the transformant cells, well known separation operations can be carried out in combination. Examples of the isolation and purification include treatment with a modifying agent such as urea or with a surfactant, ultrasonic treatment, enzyme digestion, salting out or the solvent precipitation method, dialysis, centrifugation, ultrafiltration, gel filtration, SDS-PAGE, isoelectric focusing electrophoresis, ion exchange chromatography, hydrophobic chromatography, affinity chromatography, reversed phase chromatography, and the like.

Also, the rotary motor molecule V_1-ATPase of this invention is preferably a heat resisting molecule for the sake of industrial utilization. Hence, preferably, a V_1-ATPase polynucleotide is derived from a bacterium of Thermus genus, Methanococcus genus, Sulfolobus genus, or the like, which grows at 65℃ or more. Further, use of a V_1-ATPase polynucleotide derived from the thermophile *Thermus thermophilus*, which is capable of growing even at 70°C or more is particularly preferable. The V_1-ATPase polynucleotide derived from *Thermus thermophilus* has the base sequence of SEQ ID NO:1. The V_1-ATPase polynucleotide derived from *Thermus thermophilus* encodes a complex of the polypeptide (F subunit) consisting of the amino acid sequence of SEQ ID NO:2, the polypeptide (A subunit) of SEQ ID NO:3, the polypeptide (B subunit) of SEQ ID NO:4, and the polypeptide (D subunit) of SEQ ID NO:5. Therefore, expressing the 334-4196 nt sequence of SEQ ID NO:1 by means of the aforementioned genetic engineering technology can give a heat resisting V_1-ATPase comprising of three A subunits, three B subunits and one D subunit. In addition, expressing the 1-4196 nt sequence of SEQ ID NO:1 can provide a heat resisting V_1-ATPase having one F subunit bound to the D subunit thereof.

A still another preferred mode of the rotary motor molecule V_1-ATPase of this invention has substitution of Ala residue for the 232nd Ser residue or substitution of Ser residue for the 235th Thr residue, or both, in SEQ ID NO:3, and a particularly preferred mode is the improved molecule having both substitutions (hereinafter, a molecule having both substitutions may be denoted as a "TSSA variant"). In other words, in contrast to a V-ATPase of a eukaryotic cell, the reaction of the V_1-ATPase derived from a bacterium such as *T. thermophilus* has a tendency to be interrupted during the metabolic turnover of the catalyst due to the so-called MgADP inhibition (J Biol Chem 273, 20504-20510, 1998). Normally, this ADP restriction appears within 5 minutes after ATP has been added as a substrate, and in about 10 minutes the V_1-ATPase stops ATP hydrolysis. Thus, the inventors of this application have prepared some variants and studied the ADP restriction effects. As a result, the inventors have found that the aforementioned TSSA variant continues ATP activity even for one hour after the addition of ATP as a substrate.

Still another preferred mode of the rotary motor molecule V_1-ATPase of this invention is a modified molecule where either the A or B subunit or both is fixed on a substrate. The reason is that this fixation makes it possible to efficiently transmit the rotation of the D subunit. Binding of A and/or B subunit like this on a substrate can be carried out by a variety of methods, for example using covalent bonding, but preferably a method is employed involving bonding His (hectahistidine) tag to the N terminal of A subunit and then bonding this His tag to Ni-NTA slide (Nature 386: 299-302, 1997; FEBS Letters 470: 244-248, 2000).

In another preferred mode of the rotary motor molecule V_1-ATPase of this invention, the D subunit is bound to a joint material. The term "joint material" in this case means a material for transmitting the

rotational motion of the D subunit of the V_1-ATPase to another component (e.g., a gear or a shaft of a motion engine, or the like). Also, this joint material is not for connection to another component, but can also be utilized as a "probe" or a "propeller" for observing the rotation of the V_1-ATPase. Examples of joint materials that can be utilized include a plurality of previously mentioned beads (microspheres) that are connected as seen in Embodiments described below, and a fine fiber such as actin filament (Nature 386: 299-302, 1997). This joint material can be bonded to Cys residue of the D subunit, for example, by maleimide or disulfide bonding or the like. However, the D subunit of the V_1-ATPase derived from *Thermus thermophilus*, the amino sequence of which was indicated in SEQ ID NO:5, does not have Cys residues, and thus a suitable non-Cys residue needs to be replaced by Cys residue. For this reason, in this invention, a joint material is preferably bound to Cys residue substituted for the 48th Glu residue or Cys residue substituted for the 55th Gln residue (preferably both) in SEQ ID NO:5. In addition, Cys residues other than those in D subunit (a total of nine Cys residues in A subunit, three Cys residues in B subunit) are preferably replaced by other residues (e.g., Ser residues) so that these Cys residues are not bound to the joint material.

Alternatively, a joint material can be made not to bind to D subunit, but to F subunit which binds to D subunit. In this case, for example, the 28th Ser and/or the 35th Ser residue is replaced by Cys residue in the amino acid of SEQ ID NO:2, and to these Cys residues can be bound a joint material.

Furthermore, each of the above-described V_1-ATPases can be obtained by replacing a triplet encoding the specified amino residue in a V_1-ATPase polynucleotide by means of a method using a mutation kit or

the like, the mutagenesis PCR, or a polynucleotide synthesizing method (e.g., Nucleic Acid Res. 25: 3440-3444, 1997), and then expressing this mutated polynucleotide by a genetic engineering process.

Hereinafter, the present invention will be described in terms of Examples in more detail and specifically; however, the invention is by no means limited by the Examples below.

Examples

1. Material and Method

1-1. Preparation of Proteins

The V_1-ATPase was expressed through the use of E. Coli BL21-CodonPlus-RP (Stragene) transformed with the plasmid pUCV1 that possessing the DNA sequences encoding each of the A, B, D, and F subunits of the V_1-ATPase derived from *T. thermophilus* HB8 under the control of *lac* promoter. Also, the DNA sequences encoding each of the A, B, D, and F subunits were modified to prepare the following variants (the amino acid positions correspond to SEQ ID NOS:2 to 5).

I: V_1-ATPase (A-His8-tags/ΔCys/A-S232A/A-T235S/D-E48C/D-Q55C)

(1) Binding a His tag to the N terminal of the A subunit (A-His8-tags)

(2) Substituting Ser residues for all the Cys residues of the A and B subunits (ΔCys)

(3) Substituting Ala for the 232nd Ser of the A subunit (A-S232A)

(4) Substituting Ser for the 235th Thr of the A subunit (A-T235S)

(5) Substituting Cys for the 48th Glu of the D subunit (D-E48C)

(6) Substituting Cys for the 55th Gln of the D subunit (D-Q55C)

II: V_1-ATPase (A-His8-tags/ΔCys/A-S232A/A-T235S/F-S28C/F-S35C)

(1) Binding a His tag to the N terminal of the A subunit (A-His8-tags)

(2) Substituting Ser residues for all the Cys residues of the A and B subunits (ΔCys)

(3) Substituting Ala for the 232nd Ser of the A subunit (A-S232A)

(4) Substituting Ser for the 235th Thr of the A subunit (A-T235S)

(7) Substituting Cys for the 28th Ser of the F subunit (S28C)

(8) Substituting Cys for the 35th Ser of the F subunit (S35C)

After transformed cells were suspended in 20 mM imidazole/HCl (pH 8.0) containing 0.3 M NaCl, and after the resulting suspension was heated at 65℃ for 30 minutes, the proteins unstable under heat were removed, and then the resulting material was placed into a Ni^{2+}-affinity column (Amersham) and eluted with 0.5 M imidazole/HCl (pH 8.0) containing 0.3 M NaCl. To the eluate was added a buffer, and this mixture was given ultrafiltration (VIVA-Spin, VIVA science) and was subsequently put into a RESOURCE Q column. The portion containing a V_1-ATPase was placed into Superdex 200 column (Amersham) and there the contamination-relating proteins were removed. The purified V_1-ATPase was subjected to biotinylation with more than two moles of 6-[N'-[2-(N-maleimido)ethyl]-N-piperazinylamido]hexyl D-biotinamide (biotin-$PEAC_5$-malaimide, Dojindo). The resulting substance was incubated at 25℃ for 15 minutes and then the protein was placed into a PD-10 Column (Amersham) where the unreacted reagents were removed. Biotinylation of the D and F subunits was confirmed by the western blotting technique using streptavidin-alkalinephosphatase conjugate (Amersham) (Figure 2).

1-2 Rotation Observation

Flow cell of 5μl was fabricated from two cover slips (a spacer with a thickness of 50 nm between them). The bottom glass surface was coated with Ni^{2+}-nitrilotriacetic acid, and the biotinylated V_1-ATPase (0.1-1 μM) contained in the A solution composed of a buffer (50 mM Tris-HCl, pH 8.0, 100 mM KCl, 5 mM $MgCl_2$) and 0.5% (w/v) BSA was poured into the flow cell, and His tags were bound to the glass, thereby fixing the V_1-ATPase.

The flow cell was filled with solution of beads (ϕ = 0.56 μm, Bangs Laboratories inc.) coated with 0.1% (w/v) Stereptavidin, binding some beads to the D or F subunit by biotin-streptavidine bonding. The unbound beads were removed by washing.

With the rotation of the V_1-ATPase molecule, the rotation of a bead was in ATP of specified concentration (0.2 mg/ml creatine kinase and 2.5 mM creatine phosphate ATP in the regenerating system) observed under a bright field microscope (IX70, Olympus, magnifying power 1000). Also, the state of rotation was recorded with a CCD camera. This V_1-ATPase rotation observation system is similar to F_1-ATPase rotation system (Proc Natl Acad Sci USA 98, 13649-54, 2001). Specifically, rotation about a slanted bonding, due to the beads being bound to the D or F subunit, was observed (Figure 2).

1-3. Other Assays

The protein concentrations were determined by UV measurement. The ATP hydrolytic activity was determined from the oxidation of NADH which couples pyruvate kinase and lactate dehydrogenase.

2. Results

2-1. Observation of Rotation

The two variants; the V_1-ATPase (A-His8-tags/ΔCys/A-S232A/

A-T235S/D-E48C/D-Q55C) and the V_1-ATPase (A-His8-tags/ΔCys/A-S232A/A-T235S/F-S28C/F-S35C) were observed for the rotation thereof. The two variants showed kinetics that follow the Michaelis Menten equation, with the two variants having Km of 0.3 to 0.5 mM and Vmax (turnover rate) of about 10 sec^{-1}. These values are almost the same as those of the wild type F_0F_1-ATP synthase (J Biol Chem 273, 20504-1014, 1998).

2-2. Rotation of D Subunit

When a buffer containing ATP was poured into flow cell, the rotation of a bead bound to D subunit of the V_1-ATPase was observed (Figures 3A to 3D). In flow cell, rotations of 5 to 10 beads were observed.

The rotation was in one direction, in the case of F_1-ATPase the rotation always counterclockwise viewed from the cell membrane side. In a buffer not containing ATP, one-direction rotation distinguishable from Brownian motion was not observed.

An azide is known to inhibit both ATPase activity and rotation of the F_1-ATPase (Nature 386, 299-302, 1997), but not to inhibit the ATPase activity of a V_1-ATPase (J Biol Chem 265, 21946-50, 1990). The rotation of a variant V_1-ATPase is the same as the above. This is because an azide did not affect the rotation of a V_1-ATPase in the presence of 4 mM ATP (Figure 3A and 3B) or in the presence of 0.1 mM ATP.

The average number of revolutions in the presence of 4 mM ATP was about 2.6 rps (revolutions per sec) or fewer. The average number of revolutions in the presence of 1 mM ATP was about 2.4 rps or fewer. Assuming that one revolution consumes three molecules of ATP, the revolution speed is in good agreement with the ATP hydrolysis speed observed in the bulk enzyme reaction theory (hydrolysis of about 10 ATPs per sec). Also, at 0.5 mM ATP the average number of revolutions is decreased to about 2.2 rps (Figure 3C).

2-3. Rotation of F Subunit

The rotation of a bead bound to F subunit was observed as well. Under a condition of 4 mM ATP concentration, 1 to 3 rotating beads were observed (Figure 4). The rotation direction was always counterclockwise. The revolution speed was about 2.5 rps, which was almost the same as the revolution speed of the bead on D subunit.

Industrial Applicability

As described in detail thus far, the invention of this filing provides V_1-ATPase as a novel rotary motor molecule. In addition, a variety of variant V_1-ATPases are provided which are more practical forms of this rotary motor molecule V_1-ATPase. These will greatly contribute to the fabrication of a micromachine, a nanomachine, and the like.